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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISIS INNOVATION LIMITED
2 South Parks Road
OXFORD
OX1 3UB

Patents ADP number *(if you know it)*

3998564001

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

4. Title of the invention

POLYMORPHISM II

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

STEVENS HEWLETT & PERKINS
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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Description	10
Claim(s)	2
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11. I/We request the grant of a patent on the basis of this application.

Signature Stevens, Hewlett & Perkins Agents for the Applicant Date 27 March 1998

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Kate Privett, 0171-936-2499

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POLYMORPHISM II

This invention is concerned with methods for the diagnosis of asthma and with materials and methods relating thereto.

Asthma is a disease which is becoming more prevalent and is the most common disease of childhood (1). Most asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so that factors in addition to atopy are necessary to induce the disease (2,3). Asthma is strongly familial, and is due to the interaction between genetic and environmental factors. The genetic factors are thought to be variants of normal genes ("polymorphisms") which alter their function to predispose to asthma.

Asthma may be identified by recurrent wheeze and intermittent air flow limitation. An asthmatic tendency may be quantified by the measurement of bronchial hyper-responsiveness in which an individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in air flow (PD20) or the slope of the curve between the initial air flow measurement and the last dose given (slope). Asthma is accompanied by blood eosinophilia, and eosinophils are prominent in asthmatic airways.

In the atopic response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE-coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a series of cellular events leading to the destabilisation of the cell membrane

and release of inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE.

Genetic factors underlying a disease may be identified through localisation to particular chromosomal regions by genetic linkage. Genetic linkage is established by the study of families. It relies on matching the inheritance of disease with genetic polymorphisms of known localisation (known as "genetic markers"). In a complex disease such as asthma, genetic linkage will typically localise genes to within 10 - 20 Megabases (Mb) of DNA. A region of this size may contain 350 - 700 genes, and will be too large to permit immediate identification of the disease-causing gene.

Closer localisation of disease-causing genes may be accomplished by the detection of associations between particular alleles and the disease phenotype. Over short segments of DNA, distinctive alleles of the individual polymorphisms will show non-random association with alleles of neighbouring polymorphisms. This phenomenon, known as "linkage disequilibrium" occurs over 50-500 Kilobases (Kb) of DNA. Linkage disequilibrium may be detected by the study of individuals as well as by the study of families.

Disease-causing alleles will be in linkage disequilibrium with non-functional polymorphisms from the same chromosomal segment. It is therefore possible to detect allelic association with disease from particular chromosomal segments, without identifying the exact polymorphism and gene underlying the disease state.

The detection of allelic association may therefore give information as to disease susceptibility in a particular individual. Furthermore,

allelic association is indicative of a disease-causing gene being present within 500 Kb of DNA in either direction from the allele (i.e. 1 Mb in total). Such a region may contain only 30 genes, within which the identification of the disease-causing gene is possible.

The presence of linkage disequilibrium also means that other polymorphisms may be anticipated to associate with disease, and that these additional polymorphisms will also be diagnostic of disease susceptibility in particular individuals.

Genetic associations with atopy have been demonstrated. WO 95/05481 discloses that variants of the gene encoding the β-subunit of the high-affinity receptor for IgE (FcεRIβ) are associated with atopy. It teaches a method for diagnosing atopy which is based upon the demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding FcεRIβ, located near the commencement of exon 6 of the FcεRIβ gene on chromosome 11. A further variant has also been found in which the unusual variant sequence is in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ (4).

Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (5). We have previously shown that polymorphisms within the TNF gene are associated with an increased risk of asthma (6).

The known polymorphisms do not account for all of the genetic factors which predispose to asthma. In particular, asthma is not necessarily an atopic disease. Identification of further genetic polymorphisms linked to asthma will allow the identification of children at risk of asthma before the disease has developed (for example immediately after birth), with the potential for prevention of disease. The presence of particular polymorphisms may predict the clinical course of disease (e.g. severe as opposed to mild) or the

response to particular treatments. This diagnostic information will be of use to the health care, pharmaceutical and insurance industries.

We have previously established linkage of bronchial hyper-responsiveness to chromosome 4 (8). However, this finding is of no use in diagnosis.

It has now been discovered that a genetic polymorphism known as D4S3032*5 on chromosome 4 and a nearby polymorphism known as D4S2921*13 are associated with asthmatic traits. Specifically, D4S3032*5 is associated with bronchial hyper-responsiveness and D4S2921*13 is associated with peripheral eosinophilia, both of these being traits which underlie asthma. The two polymorphisms can therefore be used as diagnostic tools.

The invention therefore provides a method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

The 1Mb region of chromosome 4 referred to flanks the D4S3032 and D4S2921 loci. Thus, the specific allele D4S3032*5, or D4S2921*13, or other unusual polymorphisms in the region which are associated with asthma, may be the subject of identification in the method according to the invention. Equally two or more such alleles may be the subject of identification, including in particular the combination of D4S3032*5 and D4S2921*13.

Current diagnostic methods involving detection at the nucleic acid level normally comprise the steps of:

(i) obtaining a suitable tissue sample from the individual;

(ii) preparing from the tissue sample a nucleic acid sample;

(iii) analysing the nucleic acid sample for the presence or absence of the relevant nucleic acid sequence, such as a specific allele.

Preferably, an amplification step is performed prior to the analysis, such that the locus at which the allele is situated is amplified. A preferred amplification technique is the PCR, although any suitable method of nucleic acid amplification may be employed.

In further aspects, the invention provides a pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921; and an assay kit comprising the pair of oligonucleotide primers.

The specific allele for identification may take the form of microsatellite repeats, which are nucleotide sequences containing short, repeated nucleotide motifs, usually a dinucleotide or a trinucleotide motif. A pair of primers which hybridize under suitably stringent conditions, to sequences at a position on either side of the microsatellite repeats, may be used to amplify the microsatellite repeats by PCR. Differences in the number of repeats are recognised by size differences in the PCR products. An allele which has a specified number of repeats and therefore a known size can thus be identified. D4S3032*5 and D4S2921*13 are examples of such alleles.

The primers employed in the method comprise nucleic acid sequences which are complementary to, or substantially complementary to unique sequences either side of the microsatellite repeats, such that only the relevant polymorphic region of the genome is amplified. The conditions under which the amplification is performed are gauged such that specific

hybridization of the primers to the flanking sequences occurs and non-specific hybridization is avoided. The hybridization conditions are suitably stringent for that purpose. Standard techniques can be used to identify an appropriate set of reaction conditions.

Typically, the PCR products are detected by means of a detectable label attached to one of the PCR primers. Alternatively another form of labeling may be used such as a labeled sequence specific probe which hybridizes to the amplified sequences. The label may be a fluorescent or other label. The PCR products are subjected to size determination, typically involving size-separation for example by gel electrophoresis, and the presence or absence of the allele of interest is determined.

It will be evident that the invention is not limited with regard to the manner in which the presence or absence of the allele of interest is determined. The labeling, detection, separation or any other aspect of the method as described here may be replaced by other suitable known techniques and reagents.

The allele for identification may be an allele other than D4S3032*5 or D4S2921*13 which is in linkage disequilibrium with D4S3032*5 or D4S2921*13 and is associated with asthma. This includes alleles of both functional and non-functional polymorphisms. Functional polymorphisms include polymorphisms within genes, usually within coding sequences of genes. Non-functional polymorphisms are polymorphisms which do not themselves cause the disease.

This invention will now be further described in the Examples section which follows. The Examples are intended to be illustrative and do not limit the scope of the invention in any way.

EXAMPLES

Description of Laboratory Testing

Subjects

Two panels of subjects have been studied.

Panel A consisted of 80 nuclear families sub-selected from an Australian population sample of 230 families (8). The panel contained a total of 203 offspring forming 172 sib-pairs. 12% of the children were asthmatic.

Panel C consisted of 87 nuclear families recruited through a child attending an asthma clinic in the Oxford region. The families contained 216 offspring (148 sibling pairs), of whom 44% were asthmatic.

Phenotyping

Bronchial responsiveness to methacholine was measured as previously described (8): the maximum dose administered was 12 μmol. The slope of the dose-response curve was calculated as (pre-dose forced expiratory volume in one second (FEV1) - last FEV1) ÷ the cumulative dose of methacholine). A constant of 0.01 was added to each measurement, to allow loge transformation when Slope was ≤ 0. Eosinophils in peripheral blood were Coulter-counted and the values $\log_e$ transformed before analysis.

Genotyping

The microsatellite markers D4S3032 and D4S2921 were typed by semi-automated fluorescent methods, as described previously (8). These markers are in close proximity at the telomeric region of the long arm of chromosome 4.

The polymerase chain reaction primer sequences for the markers were as follows:

D4S3032 5' TGA AAT TCT ATT GAC CAA TGA TGT G (SEQ ID NO: 1)

UD4S3032 5' TAG CAC CTG GAT TTA CCA TGA C (SEQ ID NO: 2)

D4S2921 5' TCC TTC AGG AAC TGG TG (SEQ ID NO: 3)

UD4S2921 5' TTA AAA ATC TAC AGA CAA GGG C (SEQ ID NO: 4)

The polymerase chain reaction conditions were as follows: The reaction volumes were 10μl, containing 50ng of genomic DNA, 200mM dNTPs, 1 x NH4+ buffer, 50ng oligonucleotide primers (forward labelled fluorescently), 0.5 to 3.0mM $MgCl_2$ and 0.2U Taq polymerase. Cycling conditions were 1 min at 95°C, 1 min at 55°C and 45s at 72°C; 28 cycles were used. PCRs were performed on an Hybaid Omnigene thermal cycler.

Electrophoresis and allele scoring were as follows:

PCR products were mlxed with a size standard (GS350 TAM) in loading buffer (80% (v/v) formamide, 20% (vlv) 50mM EDTA, 0.1% (w/v) blue dextran). Samples were denatured at 95°C for 4 min immediately prior to loading onto a 6% polyacrylamide gel and were electrophoresed at 800v for 6h on an Applied Biosystems (ABI) 313 DNA sequencer. Allele sizes were assigned using the ABI GENESCAN and ABI GENOTYPER software.

Association Analysis

Association was tested against the phenotype of bronchial hyper-responsiveness and peripheral blood eosinophil counts by the ASSOC routine of the SAGE (ver2.2) computer program.

Results

Association with D4S3032 allele 5 and D4S2921 allele 13

Each of the markers was then tested for association with the asthma phenotype. Association was seen in panel A for allele 5 of D4S3032

(D4S3032*5) and bronchial hyper-responsiveness. This allele is 145 base pairs in size, using the primers described above. Association between allele 13 of D4S2921 (D4S2921*13) and eosinophil counts were seen in both panels. This allele is 162 base pairs in size, using the primers described above. (Other suitable primers can be designed and their amplification product size determined for D4S3032*5 or D4S2921*13, using known sequence information (9).) The results of testing were as follows:

			Panel A		Panel C		Combined	
Trait	Marker	Allele	χ^2	p	χ^2	p	χ^2	p
Slope	d4s3032	5	13.56	0.0002	-	-	-	-
Eosinophils	d4s2921	13	5.00	0.03	11.17	0.0008	17.61	0.0000

The recombination fraction between D4S3032 and D4S2921 was 3%, indicating in this telomeric region that the distance between the markers is of the order of 0.5 to 1 megabase.

The results indicate that D4S3032*5 and D4S2921*13 show strong association with intermediate phenotypes underlying asthma in two diverse panels of subjects. It may therefore be inferred that a gene influencing asthma is present within 500 kilobases in either direction of D4S3032 and D4S2921.

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CLAIMS

1. A method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

2. The method according to claim 1, wherein the method comprises the steps of:

(i) obtaining a suitable tissue sample from the individual;

(ii) preparing from the tissue sample a nucleic acid sample;

(iii) analysing the nucleic acid sample for the presence or absence of the allele.

3. The method according to claim 2, wherein prior to analysis, the locus at which the or each allele is situated is amplified.

4. The method according to claim 3, wherein the amplification is by the PCR.

5. The method according to any one of claims 1 to 4, wherein the locus at which the or each allele is situated comprises microsatellite repeats of variable length.

6. The method according to claim 3 or claim 4, wherein the amplification is performed using a pair of primers for each allele, wherein each primer in a pair hybridises under suitably stringent conditions to a region either side of the microsatellite repeats.

7. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S3032*5.

8. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S2921*13.

9. The method according to any one of claims 1 to 6, wherein the alleles for identification are D4S3032*5 and D4S2921*13.

10. The method according to any one of claims 3 to 9, wherein the analysis is carried out by size separation of amplification products.

11. The method according to claim 10, wherein the primers in the pair of primers comprise the oligonucleotide sequences identified by SEQ ID NO: 1 and SEQ ID NO: 2 or substantially similar sequences, for D4S3032*5; or identified by SEQ ID NO: 3 and SEQ ID NO: 4 or substantially similar sequences, for D4S2921*13; or both of the aforementioned pairs of primers for both of the aforementioned alleles.

12. A pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 2 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

13. The pair of oligonucleotide primers according to claim 12, one of which is labeled with a detectable marker.

14. The pair of oligonucleotides according to claim 12 or claim 13, capable of hybridising under suitably stringent conditions to a region either side of a region of microsatellite repeats at D4S3032 or D4S2921.

15. The pair of oligonucleotide primers according to claim 14, comprising the oligonucleotide sequences identified by SEQ ID NO:1 and SEQ ID NO:2 or substantially similar sequences, for D4S3032*5; or the oligonucleotide sequences identified by SEQ ID NO: 3 and SEQ ID NO:4 or substantially similar sequences, for D4S2921*13.

16. An assay kit which comprises the pair of oligonucleotide primers according to any one of claims 12 to 15.

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